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The Amaryllidaceae Isocarbostyril Narciclasine Induces Apoptosis By Activation of the Death Receptor and/or Mitochondrial Pathways in Cancer Cells But Not in Normal Fibroblasts[1]

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Abstract

Our study has shown that the Amaryllidaceae isocarbostyril narciclasine induces marked apoptosis-mediated cytotoxic effects in human cancer cells but not in normal fibroblasts by triggering the activation of the initiator caspases of the death receptor pathway (caspase-8 and caspase-10) at least in human MCF-7 breast and PC-3 prostate carcinoma cells. The formation of the Fas and death receptor 4 (DR4) death-inducing signaling complex was clearly evidenced in MCF-7 and PC-3 cancer cells. Caspase-8 was found to interact with Fas and DR4 receptors on narciclasine treatment. However, narciclasine-induced downstream apoptotic pathways in MCF-7 cells diverged from those in PC-3 cells, where caspase-8 directly activated effector caspases such as caspase-3 in the absence of any further release of mitochondrial proapoptotic effectors. In contrast, in MCF-7 cells, the apoptotic process was found to require an amplification step that is mitochondria-dependent, with Bid processing, release of cytochrome *c*, and caspase-9 activation. It is postulated that the high selectivity of narciclasine to cancer cells might be linked, at least in part, to this activation of the death receptor pathway. Normal human fibroblasts appear approximately 250-fold less sensitive to narciclasine, which does not induce apoptosis in these cells probably due to the absence of death receptor pathway activation.

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Introduction

Cancer cells have an inbuilt urge to survive, so that any genetic change that favors survival against adverse conditions will be selected [1–3]. As a consequence, some tumors will survive exposure to even the most potent therapeutic agents [1–3].

Today, a large number of drugs used to fight cancer are proapoptotic. The majority of proapoptotic cytotoxic drugs currently used to treat cancer patients take advantage of cell division itself in an attempt to achieve selective action, based on the more rapid division of cancer cells compared to their normal counterparts [4]. However, major problems with these molecules persist because they are not sufficiently selective for cancer cells, resulting in toxicity to normal cells and provoking widespread and serious consequences in patients [4]. Therefore, the need to identify potent anticancer agents that target tumor cells more selectively remains.

Plants of the Amaryllidaceae family have long been known for their toxicity and medicinal properties. For instance, oil from the daffodil *Narcissus poeticus* L. was employed as treatment for cancer-related diseases in ancient Greece [5]. Among the many compounds isolated from Amaryllidaceae, hydroxylated phenantridones, also referred to as Amaryllidaceae isocarbostyrils, have long been under scrutiny due to their promising antitumor activity. Narciclasine (Figure 1*A*) was first isolated from several varieties of narcissus in 1967 [6]. Seventeen years later in 1984, pancratistatin (Figure 1*A*) was purified from the bulbs of *Pancratium littorale* (also known as *Hymenocallis littoralis*; Figure 1*A* [7]). Little is known of the mechanism of action of this family of compounds. Narciclasine was originally described as antimitotic and as displaying colchicine-like effects [6]. It was also found to be an inhibitor of peptide bond formation in eukaryotic ribosomes, given its ability to bind to the 60S ribosomal subunit and more precisely to the peptidyl-transferase center [8,9]. Furthermore, unlike many other anticancer drugs, narciclasine has been found not to interact or form a complex with DNA [10]. More recently, McLachlan et al. [11] demonstrated that pancratistatin, whose chemical structure is very close to that of narciclasine (Figure 1*A*), induced rapid apoptosis in SHSY-5Y neuroblastoma cells, accompanied by disruption of mitochondrial membrane potential $\Delta\Psi_m$. Additionally, a

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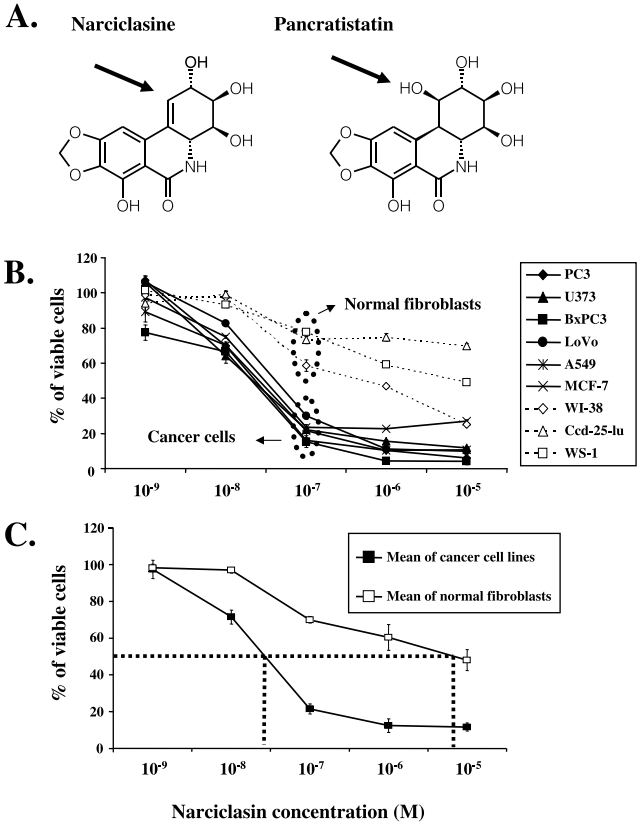

Figure 1. *Structure and cytotoxic activity of narciclasine. (A) Chemical structure of narciclasine and pancratistatin. (B) Effect of narciclasine on the overall survival of normal and tumor cells. The cytotoxic activity of narciclasine was analyzed using MTT assay, as described in the Materials and Methods section. Six tumor cells lines were tested: PC-3 prostate carcinoma, U-373 glioma, MCF-7 breast carcinoma, BxPC-3 pancreatic cancer, A-549 NSCLC, and LoVo colon cancer. To determine the selectivity of narciclasine for cancer cells, a panel of three normal human fibroblast cell lines was used: WI-38, WS-1, and Ccd-25-LU cells. (C) Cells were incubated with narciclasine for 72 hours at concentrations ranging from 1 nM to 10 μM, with semilog concentration increasing. Top graph: The effect of narciclasine on each individual cell line. Bottom graph: The mean activity of narciclasine against the six tumor cell lines compared to the mean of the three normal cell lines.*

decrease in ATP synthesis and an increase in the production of reactive oxygen species, which are indicative of a dysfunction of the mitochondrial respiratory chain, were observed in intact mitochondria incubated with the molecule [11]. Narciclasine and pancratistatin also emerged as interesting antitumor drugs in the National Cancer Institute database (http://dtp.nci.nih.gov/), with convincing *in vivo* data obtained in several mouse cancer models. The present study thus aims to characterize the mechanism of action of narciclasine when used at 1 μM *in vitro* in human normal *versus* cancer cell lines.

Materials and Methods

Narciclasine Isolation

Fresh bulbs of *Narcissus pseudonarcissus* Carlton (500 g) were homogenized, and ethanol (1 l) was added. The mixture was stirred at room temperature for 24 hours. After filtration, residue was resuspended in ethanol for 2 hours and filtered again. The two filtrates were combined, and ethanol was removed under reduced pressure to leave a residual essentially aqueous extract of ~200 ml. This was first extracted thrice with CH_2Cl_2 and then thrice with ethyl acetate. The combined ethyl acetate extracts were concentrated under reduced pressure to obtain 600 mg of the residue that was dissolved and subsequently applied to a silica gel chromatography column (particle size distribution: 40–63 μm) and eluted first with CH_2Cl_2 and then with CH_2Cl_2/MeOH (9:1). The solid residue (100 mg) obtained (R_f = 0.6 CH_2Cl_2/MeOH, 8:2) was then crystallized in methanol to obtain 46 mg of narciclasine (overall yield: 0.009%). The structure of narciclasine was confirmed after detailed analyses of ^1H, ^{13}C, distortionless enhanced polarization transfer, correlation spectroscopy, heteronuclear multiple quantum correlation nuclear magnetic resonance spectra, and mass spectra, and by comparisons with literature data.

Cell Lines and Culture Conditions

Cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Culture media, supplements, and trypsin–EDTA solution were obtained from Gibco-BRL/Invitrogen (Merelbeke, Belgium). Fetal bovine serum (FBS) was inactivated before use by heating for 1 hour at 56°C. Cells were cultured at 37°C in Falcon tissue culture dishes (Nunc Invitrogen, Merelbeke, Belgium) in an incubator containing a 5% CO_2 humidified atmosphere. The MCF-7 breast (ATCC; HTB-22) and PC-3 prostate cancer (ATCC; CRL-1435) cell lines were cultivated in RPMI 1640 (Gibco-BRL/Invitrogen; cat. no. 52400-025); the MDA-MB-231 breast cancer cell line (ATCC; HTB-26) was cultivated in DMEM–F12 (Gibco-BRL/Invitrogen; cat. no. 31330-03); and the normal human fibroblast cell line Ccd-25-Lu (ATCC; CCL-215) was cultivated in BME (Gibco-BRL/Invitrogen; cat. no. 41010-026). Culture media were supplemented with 10% FBS, 200 IU/ml penicillin, 200 IU/ml streptomycin, and 0.1 mg/ml gentamicin. In addition to cancer cell lines MCF-7 and PC-3 and the normal human fibroblast cell line Ccd-25-Lu, normal human fibroblasts WI-38 (ATCC; CCL-75) and WS-1 (ATCC; CRL-2029) and human cancer cell lines pancreatic BxPC-3 (ATCC; CRL-1687), glioblastoma U-373 (ATCC; HTB-17), non–small cell lung cancer (NSCLC) A549 (ATCC; CCL-185), and colon carcinoma LoVo (ATCC; CCL-229) were specifically used in MTT (3-[4,5-dimethylthiazol-2yl]-diphenyltetrazolium bromide) assays, as described below.

Determination of Cytotoxicity In Vitro

The effects of narciclasine on the overall proliferation rate/cytotoxicity of cancer and normal cells lines *in vitro* were determined using colorimetric MTT (Sigma-Aldrich, Bornem, Belgium) assay, as previously described [12,13]. Briefly, cells seeded in 96-well culture plates were treated for 72 hours with different concentrations of narciclasine ranging from 1 nM to 10 μM, with semilog concentration increasing. The yellow MTT product is converted to a blue formazan derivative through mitochondrial enzymatic reduction. At the end of the incubation, blue formazan is solubilized with DMSO, and absorbance at 570 nm, which is directly proportional to

the number of living cells, is determined. Experiments were carried out in sextuplicate.

Mitochondrial Membrane Potential ($\Delta\Psi_m$) Determination

$\Delta\Psi_m$ was monitored using the $\Delta\Psi_m$-specific cationic lipophilic dye JC-1 (Calbiochem; cat. no. 420200, Nottingham, UK) and flow cytometry. JC-1 staining was undertaken according to a previously published method [14,15], with minor modifications. Briefly, following treatment with narciclasine, cells were harvested and cell density was determined. One million cells were stained for 10 minutes in 1 ml of culture medium containing 10% FBS and 10 μg/ml JC-1. Cells were washed once with 10 ml of ice-cold PBS and, after centrifugation, resuspended in 1 ml of ice-cold PBS. Analysis was performed immediately on an Epics XL.MCL flow cytometer (Beckman Coulter; Analis, Suarlée, Belgium) equipped with a 488-nm argon laser. Each acquisition included 10,000 events. Cells with an intact $\Delta\Psi_m$ show high red fluorescence (channel 2, FL2). Loss of $\Delta\Psi_m$ is visualized by a drop in the red fluorescence corresponding to mitochondrial JC-1 aggregates. Green fluorescence (channel 1, FL1) is the result of monomeric cytoplasmic JC-1. To perform an analysis by fluorescence microscopy, cells were seeded in six-well plates at 30,000 cells/well. On the next day, cells were incubated with and without narciclasine (0.1–1 μM) for 18 hours. At the end of the incubation, the cells were stained with 10 μg/ml JC-1 for 15 minutes at 37°C and then washed twice with PBS before immediate analysis on a fluorescence microscope.

Preparation of Mitochondria and Determination of Cytochrome c *Release*

Mitochondria were purified using a differential centrifugation method, as previously described [16,17]. Briefly, cells were harvested, centrifuged at 500*g* for 5 minutes at 4°C, and resuspended in fractionation buffer A (10 mM Hepes pH 7.4, 0.1 mM EDTA, 1 mM EGTA, and 250 mM sucrose) supplemented with protease inhibitors. Cell disruption was performed mechanically by passing the cells through a 23-gauge needle 5 to 10 times. Homogenates were centrifuged twice at 700*g* for 10 minutes at 4°C to remove intact cells, lysed membranes, and nuclei. Supernatants were then further centrifuged at 7000*g* for 20 minutes at 4°C to pellet the mitochondria. The supernatants (cytosolic fraction) were kept for analysis. The pellets containing the mitochondria were resuspended in fractionation buffer B (10 mM Hepes pH 7.4, 5 mM KH_2PO_4, 5 mM succinate, and 250 mM sucrose) and centrifuged at 10,000*g* for 15 minutes. The resulting purified mitochondria were then resuspended in fractionation buffer B at a final protein concentration of 2 to 3 mg/ml. For complete lysis of mitochondria before Western blot analysis, preparations were incubated overnight at −80°C. As process controls, Western blot analyses were performed to measure the levels of GRP75 (Santa Cruz Biotechnology, Santa Cruz, CA), cytochrome *c* (cyt *c*; BD Pharmingen, Erembodegem, Belgium), and proliferating cell nuclear antigen (PCNA; Calbiochem) in both cytoplasmic and mitochondrial fractions. For cyt *c* release assays, aliquots from mitochondrial preparations (equivalent to 20 μg of protein) were incubated with or without narciclasine (10 μM) for 1 hour at 37°C. Mitochondria were then spun down by 15 minutes of centrifugation at 10,000*g* (4°C). The supernatants and pellets of different incubations were then investigated for the presence of cyt *c* by Western blot analysis.

DNA Fragmentation Assay

Cells (1.5×10^6) were treated with narciclasine (1 μM) for different periods of time (0, 24, 48, and 72 hours). At the end of each incubation period, intact chromatin was separated from small apoptotic DNA fragments using the method and reagents of the Suicide-Track DNA Ladder Isolation kit (Calbiochem). Purified fragments were then resolved by standard agarose gel (1.5%) electrophoresis and stained with 0.5 μg/ml ethidium bromide for visualization under UV light.

Annexin V Assay

Control and treated cells were harvested by trypsinization, resuspended in serum-containing medium to neutralize trypsin, and washed twice with PBS. Cells were then resuspended in ice-cold 1× binding buffer (Sigma-Aldrich) at a density of 10^6 cells/ml, and aliquots of 500 μl were stained with 5 μl of Annexin V (Ann V)–FITC and 10 μl of propidium iodide (PI; Sigma-Aldrich), as previously described [18]. Fluorescence was analyzed immediately on an Epics XL.MCL flow cytometer (Beckman Coulter) equipped with a 488-nm argon laser.

Western Blot Analysis and Immunoprecipitation

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.5% deoxycholic acid, and 1% NP-40) supplemented with protease inhibitors (Sigma-Aldrich). Protein concentration was quantified using the bicinchoninic acid protein assay kit (Pierce; Perbio Science, Erembodegem, Belgium). For each sample, equal amounts of proteins were subjected to SDS-PAGE and transferred onto Polyscreen PVDF membranes (NEN Life Science Products, PerkinElmer, Belgium). Membranes were blocked and incubated with primary antibody overnight in Tris-buffered saline (TBS) with 0.2% Tween-20 and 5% nonfat dry milk (or 5% bovine serum albumin, as required). After three washes of 15 minutes in TBS containing 0.2% Tween-20, blots were incubated for 1 hour with peroxidase-conjugated secondary antibody (Pierce) and then washed thrice in TBS with 0.2% Tween-20, followed by chemiluminescent detection (Pierce). For immunoprecipitation of death receptors, cells were lysed in lysis buffer containing 150 mM NaCl, 30 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 5 mM EDTA, and protease inhibitors. Equal amounts of proteins (600 μg) were immunoprecipitated overnight with 2 μg of antibody. On the next day, protein-A Sepharose beads (25 μl of a 50% vol/vol solution) were added. After 30 minutes of incubation, immunoprecipitates were washed twice with 500 μl of lysis buffer and submitted to SDS-PAGE.

The primary antibodies used in the study and their suppliers are as follows: Bid antibody (cat. no. 2002), caspase-8 (1C12; cat. no. 9746), caspase-9 (cat. no. 9502), caspase-3

(3G2; cat. no. 9668), and caspase-10 (cat. no. 9752) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The Bid antibody was used at a dilution of 1:500, whereas caspases antibodies were all used at a dilution of 1:1000. Anti–cyt *c* antibody (cat. no. 556433) was obtained from BD Bioscience Pharmingen (San Diego, CA) and was used at a 1:500 dilution. Anti-Fas (C-20; cat. no. sc-715), anti–death receptor 4 (DR4) (L-20; cat. no. 31344), and anti-GRP75 (C-19; cat. no. sc-1058) antibodies were obtained from Santa Cruz Biotechnology and used at a dilution of 1:400. Anti-PCNA (ab-1) antibody was from Calbiochem and was used at a 1:400 dilution. Rabbit polyclonal anti–endonuclease G (Endo G) (cat. no. ab9647), anti–apoptosis-inducing factor (AIF; cat. no. ab1998), and rat anti-tubulin (ab6161) antibodies were obtained from Abcam (Cambridge, UK). The anti-tubulin antibody was used at a dilution of 1:3000, whereas anti–Endo G and anti-AIF antibodies were employed at a 1:500 dilution.

Results

Cancer Cells Are Markedly More Sensitive to the Cytotoxic Effects of Narciclasine Than Normal Cells

The overall cytotoxic effects of narciclasine on a panel of six human cancer and three normal human fibroblast cell lines were assessed by MTT colorimetric assay. The data illustrated in Figure 1, *B* and *C*, reveal that narciclasine displayed significantly higher cytotoxic effects on cancer cells than on normal cells. The mean IC_{50} value (the concentration that reduces by 50% the number of viable cells after 3 days of treatment) calculated for the six human cancer cell lines was 30 nM. The corresponding mean IC_{50} value for the three fibroblasts cell lines was 7.5 μM (Figure 1*C*), indicating their markedly lower sensitivity to the cytotoxic effects of narciclasine than human cancer cells. The cytotoxic activity of narciclasine was similar in the six cancer cell lines investigated—a feature consistent with National Cancer Institute data, which reveal a mean IC_{50} value of 47 nM for the compound across a panel of 60 cancer cell lines, including doxorubicin-resistant cancer cells.

Narciclasine Induces Cancer Cell, But Not Normal Cell, Apoptosis

The potential proapoptotic effects of narciclasine on Ccd-25-Lu normal human fibroblasts and human cancer cells (MCF-7 and MDA-MB-231 breast and PC-3 prostate cancers) were evaluated. In normal lung fibroblasts (Figure 2*Aa*) treated with 1 μM narciclasine for 18 hours, no marked morphologic changes were noted (Figure 2*Ab*). In sharp contrast, when MCF-7 breast cancer cells (Figure 2*Ac*) underwent the same treatment, dramatic morphologic changes consistent with cell death were observed (Figure 2*Ad*). Ccd-25-Lu (Figure 2*Ba*), PC-3 (Figure 2*Bb*), MDA-MB-231 (Figure 2*Bc*), and MCF-7 (Figure 2*Bd*) cells treated with 1 μM narciclasine for up to 72 hours were analyzed for DNA fragmentation. Internucleosomal DNA fragmentation is a hallmark of apoptosis, with low-molecular-weight DNA fragments forming a typical ladder after electrophoresis. Although DNA laddering or smearing was not observed in Ccd-25-Lu fibroblasts (Figure 2*Ba*), clear DNA laddering was visible after only 24 hours of treatment of PC-3 (Figure 2*Bb*) and MDA-MB-231 (Figure 2*Bc*) cells and after 72 hours of treatment of MCF-7 cells (Figure 2*Bd*). The delay observed in MCF-7 cells in DNA laddering is likely to be due to the absence of caspase-3 in these cells [19]. DFF40/CAD, the main endonuclease that drives DNA fragmentation during apoptosis, forms an inactive complex in healthy cells with its inhibitor DFF45/ICAD. The latter protein needs to be cleaved in a caspase-dependent manner for DFF40 to become active [20]. Caspase-3 is thought to be the main caspase responsible for the processing of DFF45 [20].

Externalization of phosphatidyl serine, an early apoptotic event that is the result of the flipping of plasma membrane regions [21], was also investigated by means of double labeling with Ann V and PI. The proportion of cells positive for Ann V and negative for PI (early apoptotic compartment) increased markedly in PC-3 prostate cancer (Figure 2*Ca*) and MDA-MB-231 breast cancer (Figure 2*Cb*) cells treated with 1 μM narciclasine for 24 hours. Only a modest increase in the percentage of Ann V–positive to PI-negative cells (from 9.5% to 13.3%) was observed in Ccd-25-Lu fibroblasts after 24 hours of incubation with 1 μM narciclasine (Figure 2*Cc*).

Narciclasine Induces Mitochondrial Dysfunctions in Cancer Cells: Loss of $\Delta\Psi_m$ and Release of Cyt c

Mitochondrial outer membrane permeabilization (MOMP) is a crucial step in the apoptotic process and is often concomitant with a decrease in $\Delta\Psi_m$ as a direct consequence of the loss of integrity in the outer mitochondrial membrane (OMM) [22]. MOMP is lethal because it leads to the release of diverse apoptotic effectors contained in the mitochondrial transmembrane space and compromises mitochondrial energy metabolism [22]. JC-1 dye was used to measure $\Delta\Psi_m$ in untreated and narciclasine-treated cells, with accumulation of the dye into the mitochondria being directly dependent on their $\Delta\Psi_m$ [14,15]. Once inside the mitochondria, the JC-1 dye forms aggregates that fluoresce bright red. In contrast, the dye fluoresces green in the cytoplasm, where it remains in a monomeric form [14,15]. An 18-hour incubation of MCF-7 cells with 1 μM narciclasine resulted in a sharp decrease (from 90% to 38%) in the percentage of cells displaying an intact $\Delta\Psi_m$, as analyzed by flow cytometry (Figure 3*A*). Similar results were obtained in MCF-7 cells treated with 100 nM or 1 μM narciclasine for 24 hours (Figure 3*B*). A similar level of decrease in $\Delta\Psi_m$ was also observed in PC-3 prostate carcinoma cells incubated with 1 μM narciclasine for 18 hours (Figure 3*C*). No loss of $\Delta\Psi_m$ was observed after the same treatment of normal Ccd-25-Lu lung fibroblasts (Figure 3*D*).

Given that the loss of $\Delta\Psi_m$ might be the consequence of MOMP, these results prompted the determination of whether apoptosis effectors such as cyt *c* were released into the cytoplasm. Accordingly, an analysis of mitochondrial and cytoplasmic fractions isolated from untreated and narciclasine-treated (24 hours at 1 μM) MCF-7 cells was undertaken to determine the levels of apoptosis effectors Endo G, AIF, and cyt *c*. GRP75, predominantly localized in the mitochondrial

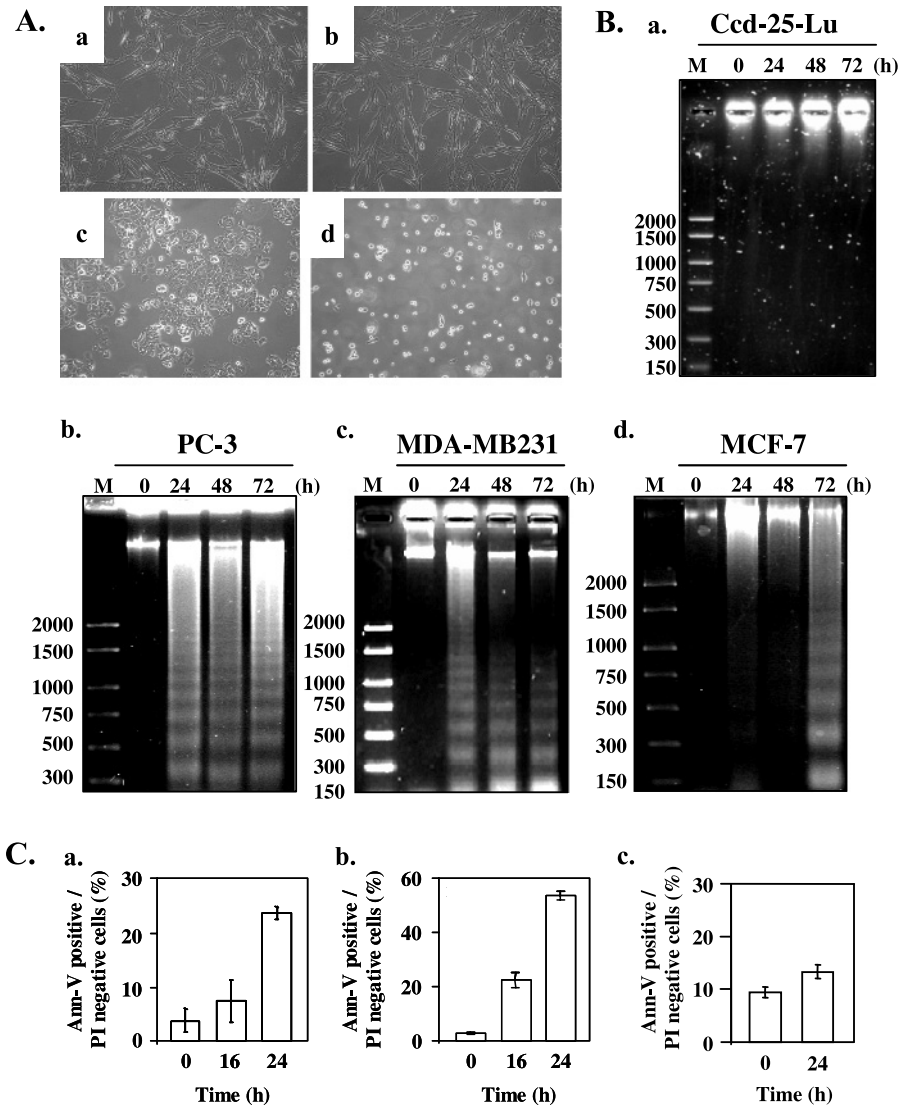

Figure 2. *Narciclasine induces apoptosis in cancer cell lines. (A) Phase-contrast images of MCF-7 breast cancer cells (c and d) and Ccd-25-LU normal human fibroblasts (a and b) untreated (a and c) or treated with 1 μM narciclasine for 18 hours (b and d). (B) DNA ladder formation on the treatment of Ccd-25-LU normal human fibroblast cells (a), PC-3 (b), MDA-MB-231 (c), and MCF-7 (d) cancer cells with narciclasine. Cells were treated for 0, 24, 48, and 72 hours with 1 μM narciclasine. At the end of the incubation, low-molecular-weight DNA fragments (< 50 kb) were extracted from an equal number of cells from each treatment and subjected to electrophoresis on a 1.5% agarose gel. Lane M, different molecular weight markers (MWMs). The sizes of the different MWM DNA bands are indicated on the left of each gel. (C) Externalization of phosphatidyl serine in PC-3 (a) and MDA-MB-231 (b) cells after treatment with 1 μM narciclasine for 16 and 24 hours, as well as in Ccd-25-LU human lung fibroblasts treated or not with 1 μM of the compound for 24 hours (c). At the end of the incubation, cells were harvested and labeled with Ann V–FITC and PI. The percentage of cells that were Ann V–positive and PI-negative (indicating early apoptotic compartments) was determined by flow cytometry analysis.*

matrix, was used as the process control [16,17]. Narciclasine provoked a decrease in the level of mitochondrial fraction cyt *c* matched by the corresponding appearance of cyt *c* in the cytoplasm (Figure 4*A*). Thus, narciclasine is able to trigger OMM permeabilization and the release of cyt *c* in MCF-7 breast cancer cells, consistent with the dramatic decrease in $\Delta\Psi_m$ previously observed. Surprisingly, AIF and Endo G were not released under the same conditions. A similar investigation was performed in PC-3 prostate carcinoma cells untreated and treated with 100 nM and 1 μM narciclasine for 24 hours. PCNA, which is localized in both the nucleus and the cytoplasm, was used as a process control [16,17]. PCNA was, as expected, found essentially in cytosolic fraction and was not readily detectable in mitochondrial fraction (Figure 4*B*). With or without narciclasine treatment, cyt *c* was found to be retained in the mitochondria (Figure 4*B*). Similarly, AIF was not released into the cytoplasm of PC-3 cells treated with the compound for 24 hours (Figure 4*C*). The presence of Endo G in PC-3 cells, as determined by Western blot analysis, could not be detected (Figure 4*C*). These results suggest that PC-3 cell mitochondria are, in all likelihood, not involved in the apoptotic process induced by narciclasine. Further testing was undertaken to ascertain whether narciclasine was able to trigger the release of cyt *c* from purified intact mitochondria. Mitochondria were purified from PC-3 cells, and their integrity was verified by Western blot analyses for GRP75, cyt *c*, and PCNA (Figure 4*D*, *upper panel*). Consistent with previous results, no release of cyt *c* from the mitochondria into the

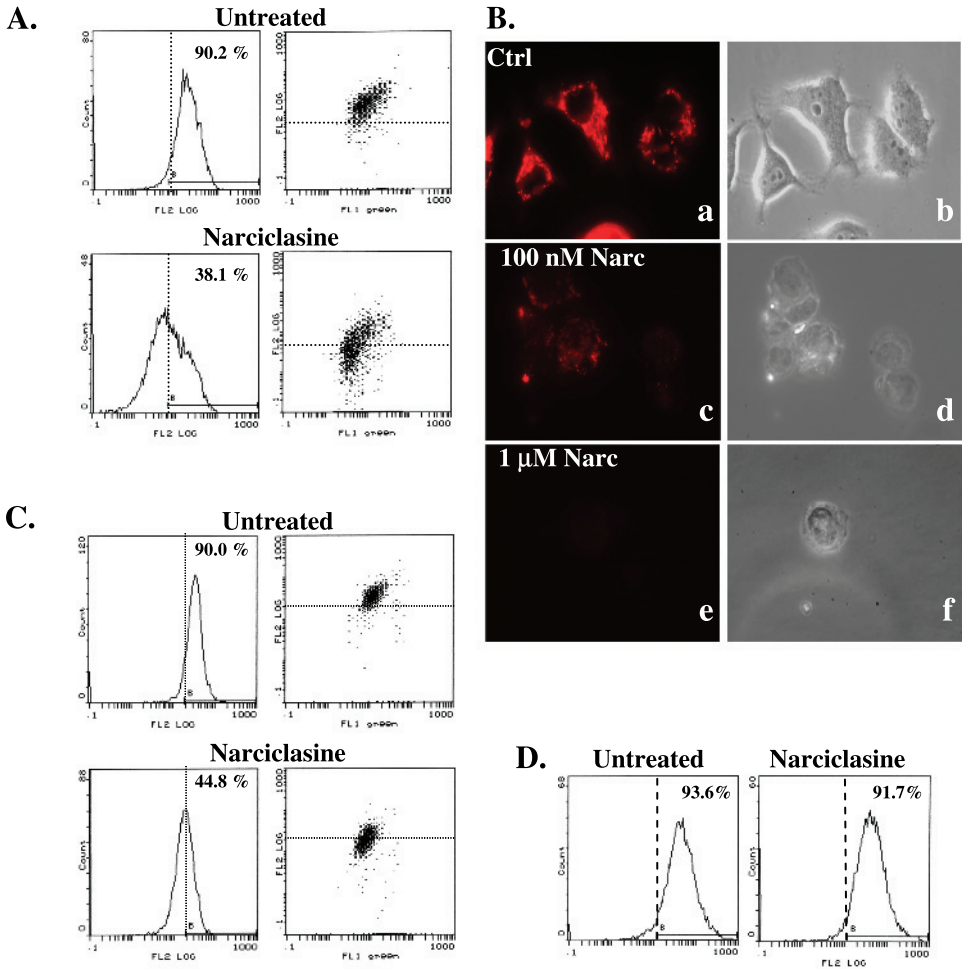

Figure 3. *Involvement of mitochondria in apoptotic cell death induced by narciclasine. (A) JC-1 staining of MCF-7 cells either untreated (top panel) or treated with 1 μM narciclasine for 18 hours (bottom panel): In each panel, the left histogram is a single-parameter analysis of the intensity of red fluorescence (specific to mitochondria with intact $\Delta\Psi_m$). The right panel is a dual-parameter analysis of red fluorescence (y-axis; FL-2)* versus *green cytoplasmic fluorescence (x-axis; FL-1). (B) JC-1 staining of MCF-7 cells and visualization by fluorescence microscopy: MCF-7 cells were left untreated or treated with 100 nM or 1 μM narciclasine for 24 hours. Images on the left (a, c, and e) depict the level of red fluorescence associated with the mitochondria. Images on the right (b, d, and f) are the corresponding bright fields. (C) JC-1 staining performed on PC-3 cells either untreated (top panel) or treated with 1 μM narciclasine for 18 hours (bottom panel). (D) Effect of narciclasine on the mitochondrial transmembrane potential of Ccd-25-LU normal human fibroblasts. Cells were incubated for 18 hours in the presence or in the absence of 1 μM narciclasine. Histograms represent a single-parameter analysis of the intensity of red fluorescence in each condition.*

supernatant after their incubation with 10 μM narciclasine for 1 hour was determined (Figure 4*D*, *lower panel*). This result was also seen in MCF-7 cells where, again, no cyt *c* was released from purified mitochondria after incubation with 0.1, 1, and 10 μM narciclasine for 1 hour (Figure 4*E*). These data collectively indicate that the involvement of the mitochondria in narciclasine-mediated apoptosis appears to be cancer cell type–specific. There is also the suggestion that the mitochondria are not the likely primary target of narciclasine and that alternative pathways may be activated first. If this is the case, then the mitochondria may only be involved in amplifying apoptotic signals and only in certain cancer cell lines.

Narciclasine Induces the Activation of Caspase-8 and/or Caspase-10 in Human Prostate and Breast Carcinoma Cells

A time course was performed after the treatment of PC-3 cells with 1 μM narciclasine, with the aim of determining the activation of caspase-3, caspase-8, and caspase-9.

Caspase-3 was activated as shown by the disappearance of procaspase-3 band (Figure 5*A*). Anti–caspase-3/clone 3G2 (Cell Signaling Technology) was the antibody used; this antibody is expected to recognize both the procaspase form and the large fragment (17/19 kDa). Unfortunately, in our hands, only the decrease of the procaspase form was observed, and we failed to detect this large processed fragment. However, the intense internucleosomal DNA fragmentation observed in the PC-3 cell line after 24 hours of treatment indicates that effector caspases are indeed activated because DNA fragmentation relies on the activity of effector caspases and, more precisely, on caspase-3. Moreover, we have also consistently observed in PC-3 cells typical membrane blebbing after treatment with narciclasine (data not shown). This morphologic change characteristic of apoptosis also relies heavily on the activity of caspase-3. It has been demonstrated that, in MCF-7 cells that are caspase-3–deficient, such morphologic change does not occur following apoptosis induction [23]. In addition, reconstitution of

caspase-3 activity (following stable transfection of caspase-3 cDNA) restores the ability of MCF-7 cells to undergo membrane blebbing [23].

Interestingly, no activation of caspase-9 was observed (i.e., no loss and, thus, no cleavage of procaspase-9) (Figure 5*A*). This correlates well with the data in Figure 4*B* indicating the absence of cyt *c* release after treatment with 100 or 1000 nM narciclasine. Indeed, in the mitochondrial pathway of apoptosis, the release of cyt *c* into the cytoplasm is a requirement for the formation of an active apoptosome complex and the subsequent processing of caspase-9 [24]. After 16 hours of treatment with narciclasine, caspase-8 is activated in PC-3 cells (Figure 5*C*). The disappearance of bands corresponding to unprocessed procaspase-8 was concomitant with an increase in processed caspase-8 (Western blots p43/p41; Figure 5*C*). These results suggest that caspase-8 itself activates caspase-3 in PC-3 prostate cancer cells. The data in Figure 5*B* indicate that anti–caspase-3 antibody is specific for the expected protein. Indeed, no band was detected in MCF-7 cells that do not express caspase-3 [25].

Caspase-8 and caspase-10 are the initiator caspases of the death receptor pathway. On activation of the death receptor, these caspases are recruited to death-inducing signaling complex (DISC), a protein complex that binds to the receptor whose major function is to permit the proteolytic autoactivation of caspase-8 and caspase-10 [26]. Further investigations in PC-3, MCF-7, and MDA-MB-231 cancer cells revealed that 16 hours after treatment with 1 μM narciclasine, caspase-8, but not caspase-10, was activated in

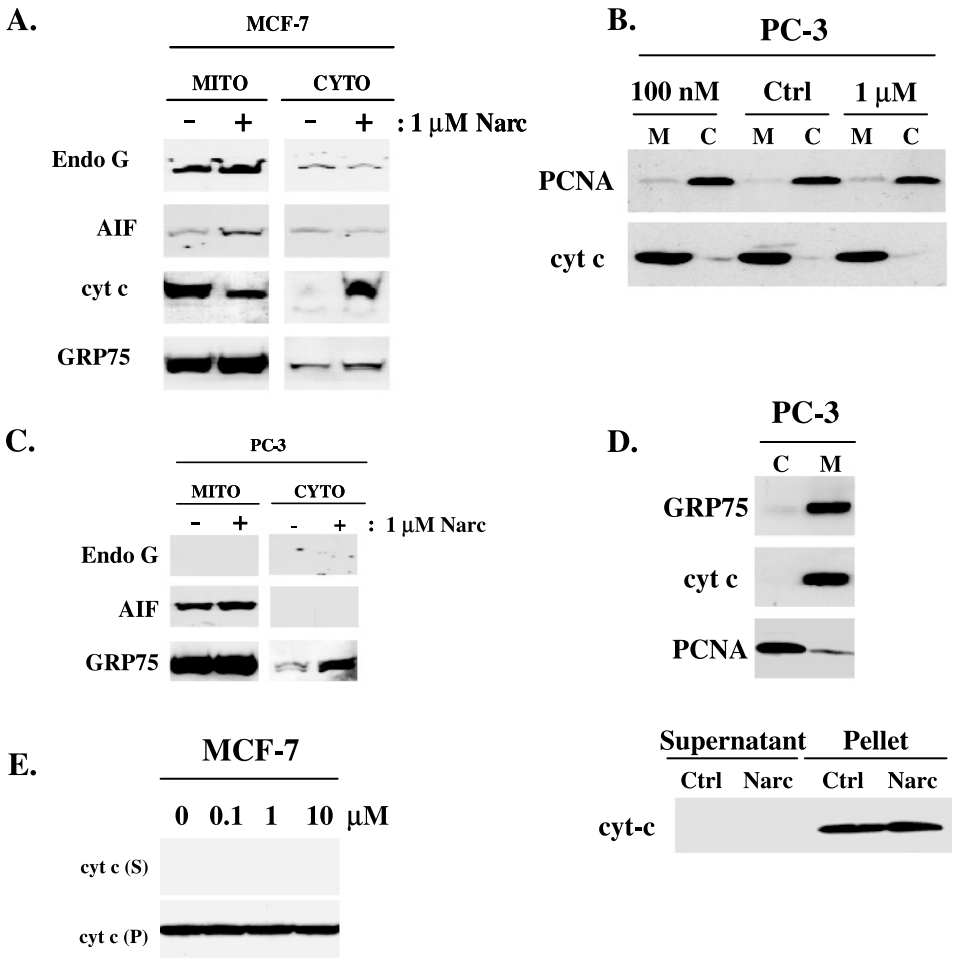

Figure 4. *Narciclasine induces the release of cyt* c *in MCF-7 but not in PC-3 cells. (A) Release of mitochondrial apoptosis effectors in MCF-7 cells untreated or treated with 1 μM narciclasine for 24 hours: At the end of the incubation period, cells were fractionated as described in the Materials and Methods section. The level of AIF, Endo G, and cyt* c *was determined by Western blot analysis in both cytosolic and mitochondrial fractions. (B) Analysis of cyt* c *release from PC-3 cells left untreated or treated with 100 nM or 1 μM narciclasine for 24 hours. Mitochondrial (M) and cytosolic (C) fractions were then isolated. Shown is an analysis of the levels of PCNA and cyt* c *in each fraction by Western blot analysis. (C) Release of AIF and Endo G in PC-3 cells left untreated or treated with 1 μM narciclasine for 24 hours. The level of AIF and Endo G was determined by Western blot analysis in both cytoplasmic and mitochondrial fractions. (D) Analysis of cyt* c *release induced by narciclasine in intact mitochondria purified from untreated PC-3 cells. Top panel: Western blots for GRP75, cyt* c*, and PCNA were performed to verify the integrity of the mitochondria. Bottom panel: Mitochondria were incubated with solvent alone (Ctrl) or with 10 μM narciclasine (Narc) for 1 hour at 37°C. At the end of the incubation, the mitochondria were pelleted by centrifugation at 10,000*g*. The level of cyt* c *was measured by Western blot analysis in both supernatants (released cyt* c*) and pellets (mitochondria). (E) Mitochondria isolated from MCF-7 cells were incubated with either solvent alone or increasing concentrations of narciclasine (0.1, 1, and 10 μM) for 1 hour at 37°C. The level of cyt* c *was determined by Western blot analysis in pellets (P) and supernatants (S) of different incubations.*

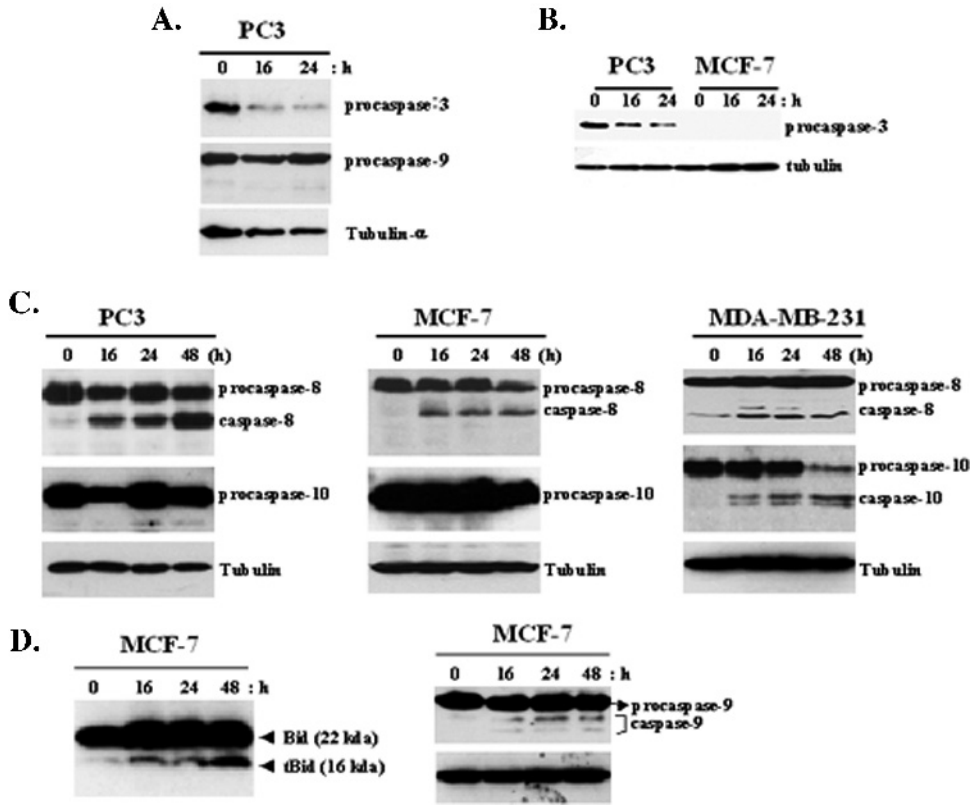

Figure 5. *Narciclasine activates caspase-8 and/or caspase-10 in human prostate and breast cancer cells. (A) Activation of caspase-3, but not caspase-9, in PC-3 cells treated with 1 μM narciclasine for 0, 16, and 24 hours. (B) Process control for the specificity of the anti–caspase-3 antibody used in this study. This was confirmed by the nondetection of procaspase-3 or caspase-3 in MCF-7 cells, a cancer cell line that is known not to express caspase-3. (C) Activation of caspase-8 and caspase-10 in PC-3, MCF-7, and MDA-MB-231 cells. Cells were treated with 1 μM narciclasine for 0, 16, 24, and 48 hours. (D) Processing of Bid and activation of caspase-9 in MCF-7 cells treated with 1 μM narciclasine for 0, 16, 24, and 48 hours.*

both PC-3 and MCF-7 cells, whereas in MDA-MB-231 cells, both caspases were found to be activated (Figure 5*C*). Procaspase-10 bands for MCF-7 could be too thick to see the decrease, but additional experiments confirmed the absence of caspase-10 activation in both PC-3 and MCF-7 cells (data not shown).

These data suggest a possible involvement of the death receptor pathway of apoptosis in the action of narciclasine. Following the formation of DISC, this apoptosis pathway involves, depending on the cell line, direct activation of effector caspases by the initiators caspase-8 and caspase-10 or, alternatively, it triggers the release of mitochondrial apoptosis effectors through the processing of Bid into an active truncated form (tBid) capable of inducing the permeabilization of the OMM. Indeed, MCF-7 cells treated with 1 μM narciclasine for up to 48 hours provoke the processing of Bid (Figure 5*D*), which correlates well with the release of cyt *c* previously observed (Figure 4*A*). Furthermore, it was found that caspase-9 under the same incubation conditions was also activated in MCF-7 cells—an activation that largely depends on the release of cyt *c* (Figure 5*D*).

Narciclasine Induces the Formation of Fas and DR4 DISC, Leading to the Recruitment of Caspase-8

The possible recruitment of caspase-8 by death receptors was investigated. PC-3 prostate cancer cells were left untreated or treated with 1 μM narciclasine for 6 hours, and then immunoprecipitation was performed on cell lysates using anti-FAS, anti-DR4, anti-DR5, and anti-TNFR1 antibodies. Subsequent Western blot analysis revealed that caspase-8 was recruited by the death receptors FAS and DR4. In narciclasine-treated cells but not in untreated cells, processed caspase-8 (p43/p41) was found to exist in a complex with both FAS and DR4, indicating the formation of DISC at the level of these two receptors (Figure 6*A*). Straight Western blots for caspase-8, FAS, DR4, and tubulin confirmed that caspase-8 was already processed after 6 hours of treatment and that an equal amount of protein had been used in different immunoprecipitations (Figure 6*A*). In contrast, no recruitment of caspase-8 by DR5 and TNFR1 was found (data not shown). Corresponding experiments performed in MCF-7 breast cancer cells and Ccd-25-Lu normal lung fibroblasts (Figure 6, *B* and *C*) revealed the recruitment of caspase-8 by FAS and DR4 in MCF-7 cells (Figure 6*B*)—although weaker than that observed in PC-3 cells—but no recruitment in Ccd-25-Lu lung fibroblasts (Figure 6*C*).

Discussion

Our results indicate that narciclasine shows potent *in vitro* cytotoxic activity against all the six cancer cell lines investigated. In contrast, normal human lung fibroblasts appear to

be markedly less sensitive to the molecule, with a mean cytotoxic IC_{50} value of 7.5 μM compared to 30 nM for the cancer cell lines. Additionally, based on comparative evaluations assessing morphologic changes, following internucleosomal DNA fragmentation or the externalization of phosphatidyl serine in normal fibroblasts and human cancer cells, the proapoptotic effects of narciclasine at concentrations of up to 1 μM were confined to cancer cells. These results collectively emphasize the marked selectivity of narciclasine for cancer cells.

Narciclasine indeed causes internucleosomal DNA fragmentation and flipping of phosphatidyl serine (hallmarks of apoptotic processes) in a manner similar to that previously reported for pancratistatin, a close structural analog (Figure 1*A*). McLachlan et al. [11] reported that pancratistatin triggers marked apoptosis in neuroblastoma cell populations, accompanied by the disruption of $\Delta\Psi_m$, DNA fragmentation, and increased reactive oxygen species production [11]. To understand the mechanism by which narciclasine induces apoptosis, the role of the mitochondria was evaluated. The mitochondria contain in their transmembrane space a variety of proapoptotic effectors such as cyt *c* [27], AIF [28], Smac/Diablo [29,30], Endo G [31], and Htra2/Omni [32], as well as several procaspases [33]. During apoptosis, these proteins are released from the transmembrane space into the cytoplasm on MOMP. In MCF-7 cells, an 18-hour incubation with 1 μM narciclasine leads to a severe decrease in $\Delta\Psi_m$. Cyt *c* was also released from the mitochondria into the cytoplasm, indicating that narciclasine causes a loss of integrity of the OMM. In PC-3 prostate carcinoma cells, although a decrease in $\Delta\Psi_m$ was observed, the magnitude of the decrease was reduced compared to that in MCF-7 cells. In addition, no release of cyt *c* was observed after 24 hours of incubation with narciclasine. Furthermore, when narciclasine was incubated at concentrations of up to 10 μM with intact mitochondria purified from MCF-7 or PC-3 cells, it failed to trigger MOMP or the release of cyt *c*. Collectively, these data suggest that the involvement of the mitochondria in

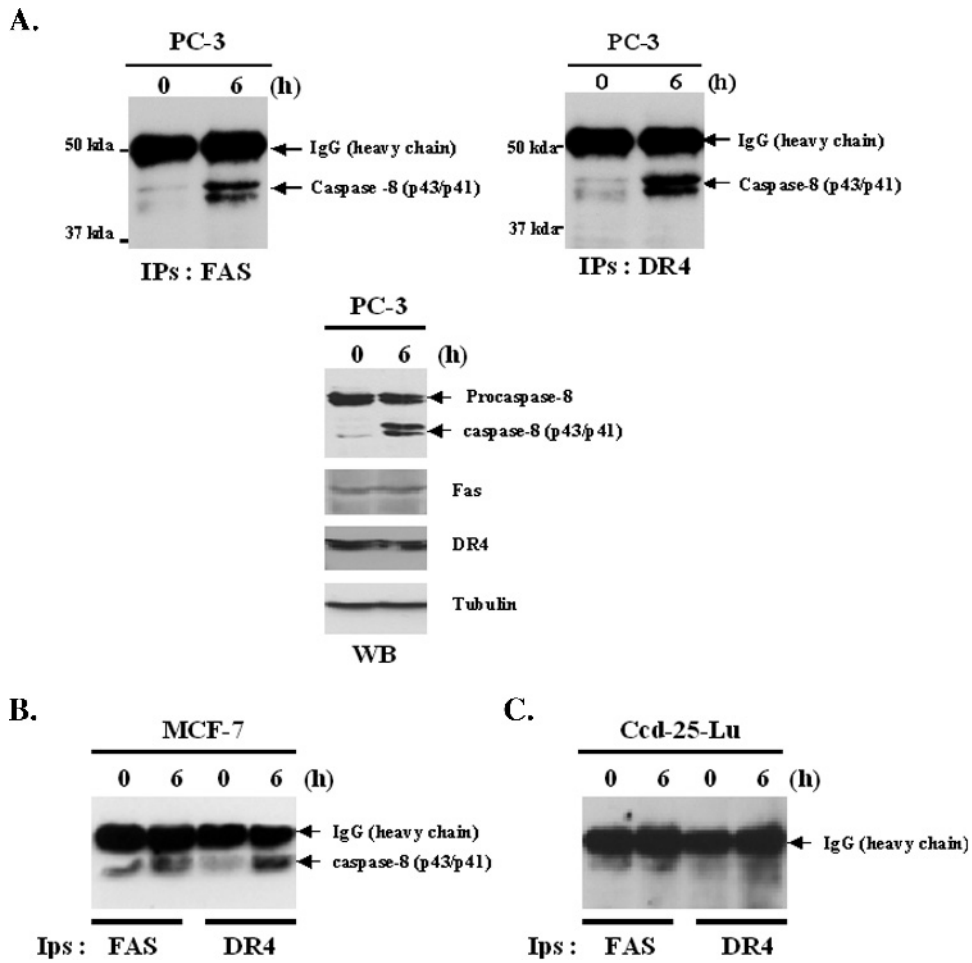

Figure 6. *Activation of the death receptors FAS and DR4 by narciclasine. (A) PC-3 cells were left untreated or treated with 1 μM narciclasine for 6 hours. At the end of the incubation, protein extracts were prepared and immunoprecipitated with anti-FAS (left panel) or anti-DR4 (right panel) antibody. After the isolation of the protein complex and electrophoresis, Western blot analysis of caspase-8 was performed. The input gel (bottom panel) shows straight Western blots for caspase-8, FAS, DR4, and tubulin aimed to verify whether an equal amount of material was used in each immunoprecipitation. (B) Immunoprecipitation of the DR4 and FAS receptors followed by detection of caspase-8 in MCF-7 cells untreated or treated with 1 μM narciclasine for 6 hours. (C) Immunoprecipitation of the DR4 and FAS receptors followed by detection of caspase-8 in Ccd-25-Lu normal human lung fibroblasts untreated or treated with 1 μM narciclasine for 6 hours.*

narciclasine-mediated apoptosis is cell type–specific and not necessarily the result of a direct action of the molecule on these organelles. Indeed, there exist two main pathways of apoptosis: the mitochondrial pathway and the death receptor pathway, also called the extrinsic pathway [34]. Death receptors belong to the tumor necrosis factor (TNF) superfamily of receptors and share in common a distinctive cytoplasmic domain called the death domain. This group of receptors includes FAS and DR3, which interact with Fas-L, DR4, and KILLER/DR5 that binds TNF-related apoptosis-inducing ligand (TRAIL), as well as TNFR1 and DR6 [34]. The death receptor pathway of apoptosis has been repeatedly involved in chemotherapy-induced cell death. This has been well described for the antitumor molecule etoposide, which largely relies on this pathway to induce apoptosis [34]. DNA-damaging agents can sensitize cells to this apoptosis pathway by modifying the expression of genes that regulate it. For instance, DR4, DR5, and FAS are p53-responsive genes [35,36]. In the three tumor cell lines in which it was examined in this study (MCF-7, PC-3, and MDA-MB-231), activation of the initiator caspases of the death receptor pathway, namely, caspase-8 and/or caspase-10, was observed. Activation of death receptors leads to the formation of DISC. The adaptor FADD binds to the death domain (DD) in the cytoplasmic portion of the receptor and subsequently recruits caspase-8 and caspase-10. These initiator caspases then undergo proteolytic autoactivation and are eventually released from DISC [34]. Our data reveal that narciclasine treatment of PC-3 prostate and MCF-7 breast carcinoma cells results in the activation of the death receptors FAS and DR4. By immunoprecipitation, it was possible to show the recruitment and activation of caspase-8 at both receptors. With regard to FAS, two types of cells can be distinguished (Figure 7). In type I cells, a high amount of procaspase-8 is recruited to DISC, leading to the activation of caspase-8 at a level sufficiently high to directly initiate a downstream caspase cascade (Figure 7) [34]. However, in type II cells, the recruitment and activation of caspase-8 at DISC, as well as the amount of formed DISC, are lower and insufficient to trigger the caspase cascade on its own (Figure 7). In type II cells, the apoptotic process requires a mitochondria-dependent amplification step (Figure 7) [34]. Thus, it appears that PC-3 prostate carcinoma cells behave on treatment with narciclasine-like type I cells: A marked recruitment of caspase-8 at FAS DISC was observed, concomitant to the processing of caspase-3 (Figure 7). In addition, no release of proapoptotic mitochondrial effectors or activation of caspase-9, whose processing is largely dependent on the release of cyt *c*, was observed. In contrast, narciclasine-induced apoptosis clearly involved the mitochondria in MCF-7 cells. Bid cleavage was also observed together with the release of cyt *c* and the subsequent activation of caspase-9, showing that MCF-7 cells react like type II cells in response to narciclasine (Figure 7).

It should be noted also that TRAIL induces programmed cell death in many tumor cells, but not in most normal cells, including normal human lung fibroblasts [37,38]. In addition, normal human lung fibroblasts do not undergo apoptosis in response to TNF-α or an agonistic anti-Fas antibody [39].

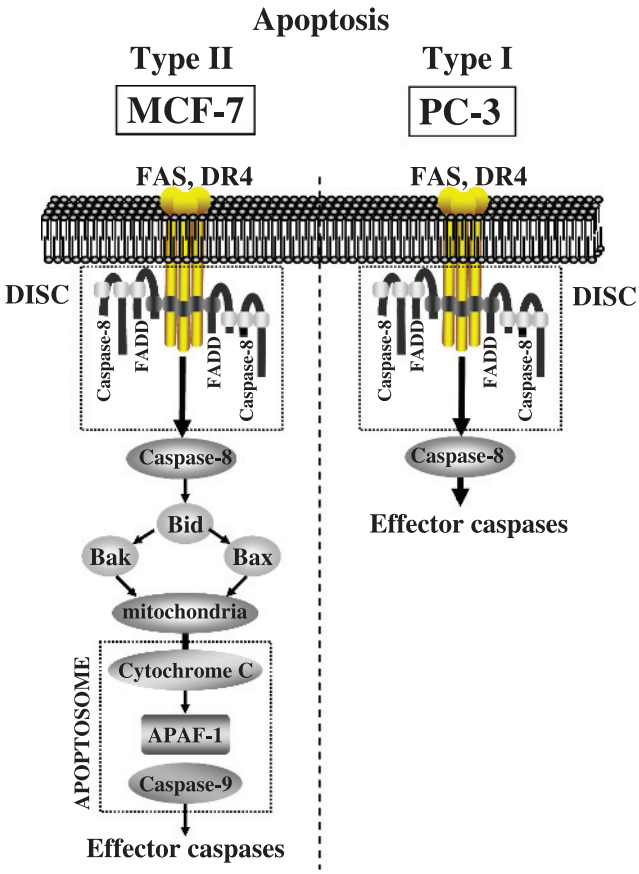

Figure 7. *Model summarizing the hypothesis on narciclasine-mediated apoptosis. After the formation of FAS and DR4 DISC and the recruitment of caspase-8, downstream signaling pathways are cell type–dependent. In PC-3 cells (type I), apoptosis proceeds by the direct activation of effector caspases by caspase-8. In contrast, MCF-7 cells (type II) amplify apoptotic signals by triggering the mitochondrial pathway, as these cells lack caspase-3 [19].*

In conclusion, Amaryllidaceae isocarbostyrils, such as narciclasine and pancratistatin, appear to be an attractive family of molecules with respect to cancer chemotherapy. Both pancratistatin [11] and narciclasine have potent *in vitro* anticancer activity and good selectivity, with tumor cells being markedly more sensitive than normal cells. Our present report emphasizes the role of the death receptors DR4 and FAS in narciclasine-mediated apoptosis.

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